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Degradation of naphthalene by *Pseudomonas* sp. strain KR3 investigated by flow calorimetry ☆

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Abstract

Biotic degradation techniques are gaining importance in decontaminating soils and waste water from organic pollutants. One class of organic pollutants of much interest are polyaromatic hydrocarbons (PAH), ubiquitous substances some of which show a high carcinogenic and mutagenic potential and acute toxicity. Although they usually possess a high thermodynamic stability they can be degraded by microorganisms. In our investigations of PAH degradation we used naphthalene as a model substance because of its high solubility.

The degradation of naphthalene at 25°C in liquid culture by *Pseudomonas* sp. strain KR3 was observed with a Thermometric microcalorimeter equipped with a flow cell. Bacterial cultures from a fermentor were pumped through this measuring cell via tubing.

Parallel to the microcalorimetric investigations the amount of degraded naphthalene and the generation of metabolites were observed by HPLC analysis, and biomass production and oxygen consumption were measured.

On degrading naphthalene, *Pseudomonas* sp. strain KR3 generated various metabolites at the same time. Only some of the metabolites had their maximum concentration synchronous to the maximum heat flow rate.

Anabolic processes were delayed compared to the consumption of the substrate.

A good correlation between calorimetric results and oxygen consumption was found.

A relationship between a strong decrease in heat production after the main heat flow rate peak and inhibition of metabolic processes by lack of oxygen could be excluded. Decrease in

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the heat flow rate is assumed to be due to lowered gas exchange in the calorimeter tubes, i.e. the inhibited degassing of carbon dioxide.

Calculation of an envelope for the heat flow rate is useful to improve experimental results.

Keywords: Calorimetry; Flow calorimetry; Naphthalene; PAH; *Pseudomonas*

1. Introduction

Polyaromatic hydrocarbons (PAH) are ubiquitous substances. Many of them show an acute toxicity. More than half of the PAH investigated until now show a high carcinogenic or mutagenic potential [1]. PAH are synthesized in a natural way by microorganisms, algae, fungi and higher plants or by volcanic and geochemical activities and fires. However, the main source of PAH in the environment is anthropogenic activity. Important origins of PAH are incomplete high temperature processes [2].

Because their water solubility is low and PAH are lipophilic, they are enriched in sediments and urban soils. A prediction of the real effects of PAH on man and the environment under natural conditions is very difficult, because of their ubiquity in air, food and water, lifelong exposition and synergistic effects with other xenobiotica.

In spite of their high thermodynamic stability, photooxidation is the most important process of abiotic degradation of PAH in surface waters and air. Furthermore they are oxidized by ozone and compounds of chlorine, nitrogen and sulfur oxides [3].

Many PAH-degrading algae, fungi and bacteria are known today. The rate of microbial degradation decreases with increasing molecular weight or decreasing solubility.

Strains able to use PAH having more than three condensed aromatic rings as the only carbon and energy source are still unknown. However, some microorganisms are able to use higher PAH, up to 5 rings, by cometabolism [4].

For sanitation of contaminated waters and soils, biotic processes are of great importance today. Optimizing degradation conditions like content of oxygen, water and minerals or the accessibility of xenobiotica for autochthonous microorganisms is a requirement for mineralization of PAH or degradation to nontoxic products. Combination with physico-chemical processes can be useful.

Microcalorimetry should be a powerful method to observe and optimize degradation processes of PAH by bacteria isolated from contaminated soils.

For our microcalorimetric investigations of aerobic degradation of PAH we used naphthalene as a model substance. It is noncancerogenic and nontoxic to man, highly soluble and completely degradable by many microbial strains. Metabolism is similar to that of other PAH.

2. Materials and methods

2.1. Calorimeter

Calorimetric measurements were performed with the 2277 Thermal Activity Monitor, a heat-conduction calorimeter with Peltier elements manufactured by Thermometric AB, Sweden. Flow measurements had a detection limit of 0.5 μW and a reproducibility of 0.2%.

2.2. Chemicals

Reagent grade naphthalene was purchased from Fluka AG, Switzerland. Reagent-grade inorganic salts and water for HPLC were obtained from Merck, Germany. Acetonitrile for HPLC was purchased from Riedel-de-Haën, Germany.

2.3. Culture

Pseudomonas sp. strain KR3 was isolated by K. Rehmann, Institute of Ecological Chemistry, GSF Neuherberg. The strain degrades naphthalene completely.

2.4. Growth conditions

Organisms were grown at 25°C in a mineral medium. The composition per liter was: KH_2PO_4, 1.44 g; K_2HPO_4, 2.94 g; $(NH_4)_2SO_4$, 0.65 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; NaCl, 0.1 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; $FeCl_3 \cdot 6H_2O$, 0.01 g and trace elements. The naphthalene (0.2 g); the only carbon and energy source, was sterilized and added separately. The reactor was stirred and aerated with humidified air.

Pure cultures were maintained on mineral medium agar slants containing 1.5% agar. Naphthalene was added on the punt of the Petri dishes and consumed by the organsims via the gas phase.

2.5. Cell growth

Growth of culture was estimated by measuring the optical density at 550 nm in an SLT Easy Reader Spectra Plus 400 ATC and by dry gravimetric determinations.

2.6. HPLC analysis

Amounts of naphthalene and metabolites were determined by an HPLC (Gynkotek, Germering, Germany) with an ODS column (Bischoff, Leonberg, Germany). Samples were injected directly after centrifugation. Separation was achieved with an acetonitrile/water (70:30, vol/vol) isocratic solvent system.

2.7. Measurement of oxygen consumption

The naphthalene-containing fermentor was inoculated and strongly aerated. Then the air stream was stopped for about 10–20 min and the decrease in oxygen content

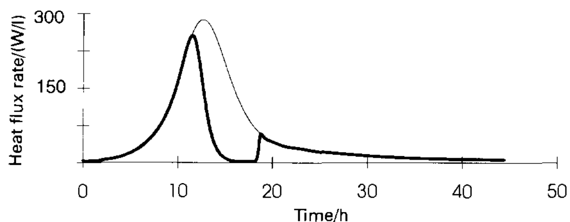

Fig. 1. Microcalorimetric heat flow rate curve (W l^{-1}) of the degradation of 200 mg l^{-1} naphthalene: ——, experimental data; —, calculated envelope.

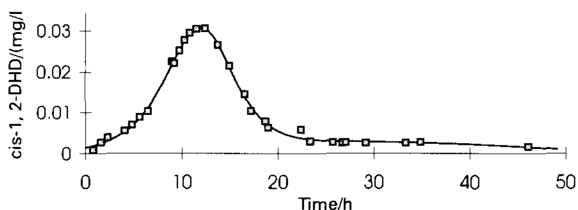

Fig. 2. Plot the concentration course (mg l^{-1}) of *cis*-1,2-dihydroxydihydronaphthalene.

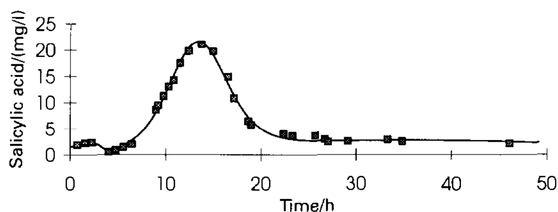

Fig. 3. Plot of the concentration course (mg l^{-1}) of salicylic acid.

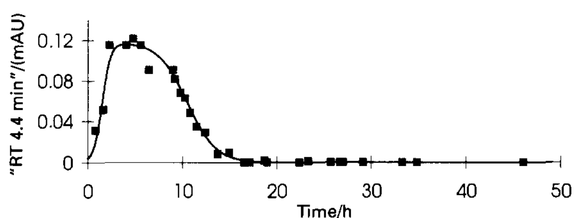

Fig. 4. Plot of the concentration course (mAU) of an unidentified metabolite with a retention time of 4 min.

was measured with an oxygen electrode EO 96 (WTW, Weilheim, Germany). Then aeration was started again. This procedure was repeated along the heat flow rate curve of the experiment. From the decrease in the oxygen concentration and the appropriate time for each measuring phase, an average oxygen consumption was calculated.

3. Results and discussion

3.1. Correlation of heat flow rate to metabolic processes

Microcalorimetric and HPLC measurements were carried out in parallel. About every hour, samples were taken and injected.

In total, twelve metabolites were detected. Only some of them could be identified. The plots of the chromatogram areas of the various metabolites during the experiment were compared to the shape of the calorimetric curve.

Some metabolites appeared simultaneously with the heat flow rate, with deviation of the maxima of only a few hours, e.g. the first metabolite of the metabolic pathway, *cis*-1,2-dihydroxydihydronaphthalene. Salicylic acid was perfectly synchronous with the calorimetric curve but showed an induction phase covering the first 5 h. Other compounds had a maximum enrichment in the first phase of the experiments or some hours after maximum thermal activity (Figs. 1–4).

Increase in optical density did not start before the heat flow rate maximum was almost reached and most of the heat dissipated. This result was contrary to well-known experiments with sugars, alcohols, cellulose and other primary natural products which showed a growth of microorganisms parallel to substrate consumption and heat production [5–7]. Anabolic processes using PAH were only possible with an increased content of low molecular weight metabolites. By conducting microcalorimetric and HPLC experiments in parallel a strong heat output and

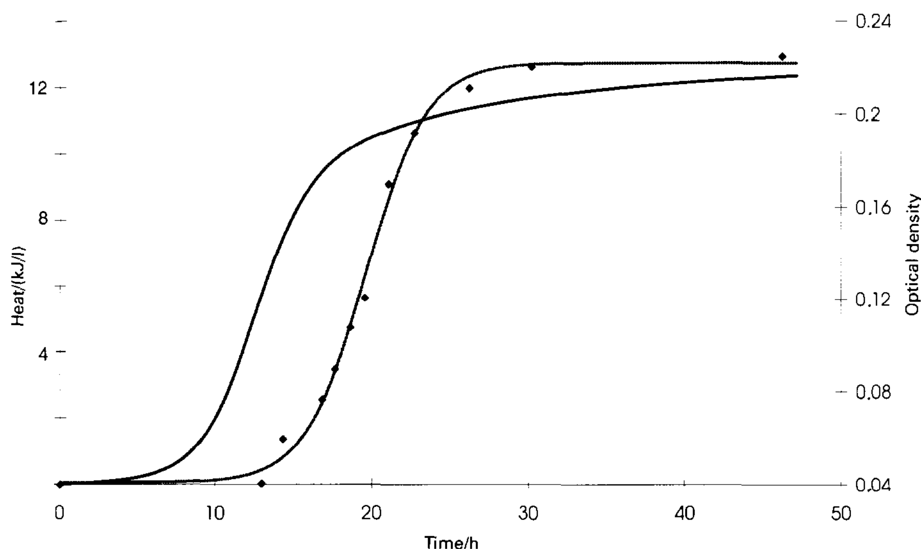

Fig. 5. Microcalorimetric heat production curve ($J\ l^{-1}$) and biomass production (optical density) of degradation of 200 mg l^{-1} naphthalene: —, heat production; ◆, optical density.

detectable amounts of various metabolites were found although no cellular growth was measurable by the photometric method. Processes of adaptation to substrate and degradative conditions, degradation of the carbon source to low molecular weight products, and establishment of energy storage, all occurred before anabolism set in. A shift in the beginning of the exponential phase from the microcalorimetric to photometric curve of about 7 h was observed (Fig. 5).

In a first experiment the heat flow rate showed a diphasic curve (Fig. 1). The second maximum could not be explained by synchronous formation and consumption of metabolites. It was not possible to reproduce it in each experiment even under the same experimental conditions. Occasionally only a shoulder of the main peak was found.

Nearly ten years ago there were attempts to interpret this phenomenon found with other substrates [8]. Hölzel et al. [9] interpreted the second maximum as an artefact resulting from the decrease in oxygen content in the tubes between fermentor and measuring cell, and the synchronous reduction of metabolism. Decreasing metabolism and oxygen consumption should start a phase with sufficient oxygen in the measuring cell. For inhibited metabolism, an unlimited envelope was calculated [9]. Considering this idea we calculated results from different experiments by means of such an envelope again and received lower standard deviations (Fig. 1).

3.2. Influence of the decrease in oxygen and increase in carbon dioxide partial pressure on the heat flow rate curve

To check the applicability of the theory of a decreasing oxygen concentration to our experiments the oxygen consumption in the fermentor was measured parallel to the calorimetric curve and the results were compared.

For a flow of 60 ml h^{-1} or about 4 min residence time, a maximum of oxygen consumption synchronous to the maximum of heat flow rate was found (Fig. 6). It must be taken into account that, because of the slow fluid motion of the medium to the electrode, the measured value in oxygen-saturated water was about 15% too low, so that the real oxygen concentration should be higher. The measured oxygen consumption calculated from the integral under the appropriate curve gave only 30% of the expected value. Therefore, the method used is not exact, but shows the temporal course of the oxygen consumption curve compared to the heat flux.

The heat flow rate was not observably affected by a measured decrease of oxygen concentration down to 2 mg l^{-1} in the medium during the measuring phase. No change in the curve shape was detected (Fig. 6).

In another experiment, the oxygen concentration at the end of model flow tube with the same dimensions as the calorimeter tubes from the fermentor to the end of the measuring cell (1250 mm Teflon, 1320 mm steel) was measured. At no point of the heat flux curve was oxygen completely consumed. The minimum measured concentration was 1.8 mg l^{-1}. Furthermore, the error of the electrode at low flow rate has to be considered.

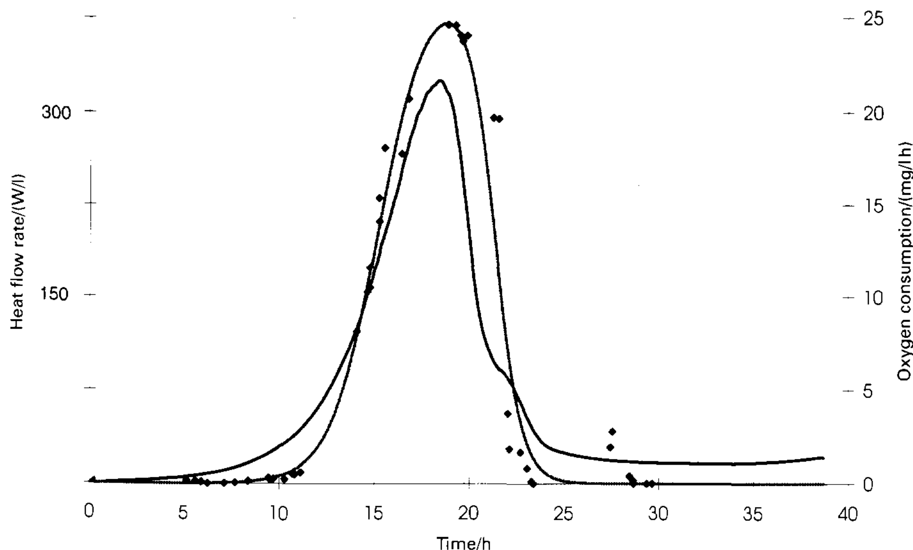

Fig. 6. Microcalorimetric heat flow rate curve (W l^{-1}) and oxygen consumption (mg l^{-1} h^{-1}) of degradation of 200 mg l^{-1} naphthalene: —, heat flow rate; ♦, oxygen consumption.

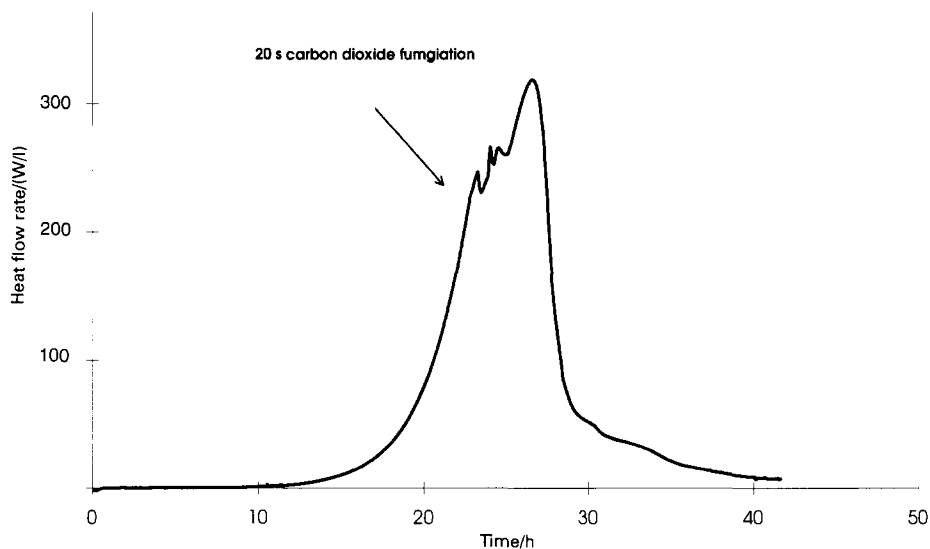

Fig. 7. Influence of carbon dioxide fumigation for 20 s on the heat flow rate curve (W l^{-1}) of naphthalene degradation.

These results demonstrated that the two-phase shape of the heat production curve is not due to the lack of oxygen at a high metabolic activity.

Differences in the microbial activity between the fermentor and the flow tubes can only be caused by gaseous substances because concentrations and effects of metabo-

lites and substrate should not differ between fermentor and measuring place. But only in the fermentor gases can exchange between medium and gas phase take place. Gas generated in the tubes, such as carbon dioxide, has to be dissolved in the liquid. Therefore the observed effect should depend on an increased partial pressure of gaseous products.

Higher partial pressure of carbon dioxide affects the activity of microorganisms. Growth inhibition of *Pseudomonas fluorescens* in a mineral medium of between 60 and 70% by a CO_2 partial pressure of 200–400 mbar has been described [10]. A low concentration of CO_2 is needed for biosynthesis of essential carboxylation products not presented in a mineral medium. At higher concentrations an influence on decarboxylation and inhibition of enzyme systems is probable [10].

An estimation of carbon dioxide production from Fig. 6 and the stoichiometric equation of naphthalene combustion ($C_{10}H_B + 12O_2 \rightarrow 10CO_2 + 4H_2O$) gives a consumption of 52 μmol O_2 l^{-1} in 4 min for the maximum oxygen consumption of about 25 mg l^{-1} h^{-1}. An equivalent 43 μmol CO_2 l^{-1} are generated. This small amount of carbon dioxide should cause a partial pressure of about 1 mbar. Even if the uncertainty of the oxygen measurement is considered, no strong influence of the carbon dioxide on pH and microbial activity should be expected. Nevertheless, following fumigation of the fermentor with carbon dioxide for only a few seconds, we detected a high sensitivity of the bacterial culture to CO_2 (Fig. 7). So the influence of lowered carbon dioxide degassing on the results of microcalorimetric measurements has to be taken into account. Although the concentration of CO_2 in the measuring tubes is low, we still hold it at least in part responsible for the decrease in metabolic activity during measurement.

Originally the calculation of an envelope of unlimited metabolism was developed for an inhibition of metabolism by lack of oxygen; this method seems to be also applicable to any inhibition occurring in the course of microcalorimetric flow measurements, thereby minimizing errors caused by the experimental method.

References

[1] J. Jacob, W. Karcher and P.J. Wagstaffe, Fresenius Z. Anal. Chem., 317 (1984) 101.

[2] A.E. McElroy, J.W. Farrington and J.M. Teal, in U. Varanasi (Ed.), Metabolism of PAH in the aquatic environment, CRC Press, Boca Raton, FL, p. 1.

[3] R. Jaffé, Environ. Pollut., 69 (1991) 237.

[4] D. Groenewegen and H. Stolp, Zentralkl. Bakteriol. Parisitenkd. Infektionskr. Hyg. Abt. Orig. B, 162 (1976) 225.

[5] R. Brettel, I. Lamprecht and B. Schaarschmidt, Radiat. Environ. Biophys., 18 (1980) 301.

[6] R. Brettel, I. Lamprecht and B. Schaarschmidt, Thermochim. Acta, 49 (1981) 53.

[7] R. Brettel, I. Lamprecht and B. Schaarschmidt, Eur. J. Appl. Microbiol. Biotechnol., 11 (1981) 212.

[8] I. Lamprecht, B. Schaarschmidt and J. Siemens, Thermochim. Acta, 94 (1985) 129.

[9] R. Hölzel, C. Motzkus and I. Lamprecht, EUROSTAR Munich, 1993, Poster presentation.

[10] U. Onken and E. Liefke, Adv. Biochem. Engin. Biotechnol., 40 (1989) 137.